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Development of Real-Time RT-PCR for the Detection of Avian Influenza Virus

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SUMMARY. A real-time reverse transcriptase/polymerase chain reaction (RRT-PCR) assay was developed using hydrolysis probes for the detection of avian influenza virus (AIV) and the H5 and H7 subtypes. The AIV specific primers and probes were directed to regions of the AIV matrix gene that are conserved among most type A influenza viruses. The H5 and H7 primers and probes are directed to H5 and H7 hemagglutinin gene regions that are conserved among North American avian influenza viruses. The sensitivity and specificity of this RRT-PCR assay was compared to virus isolation (VI) in chicken embryos with 1550 clinical swab samples from 109 live-bird markets (LBMs) in New York and New Jersey. RRT-PCR detected influenza in samples from 61 of 65 (93.8%) of the LBMs that were the sources of VI positive samples. Of the 58 markets that were positive for H7 influenza by hemagglutination inhibition assay, RRT-PCR detected H7 influenza in 56 markets (96.5%). Too few H5 positive samples were obtained to validate the H5 RRT-PCR assay in this study. Although RRT-PCR was less sensitive than VI on an individual sample basis, this study demonstrated that the AIV and H7 RRT-PCR assays are good tools for the rapid screening of flocks and LBMs.

RESUMEN. Desarrollo de una prueba de transcriptasa reversa—reacción en cadena por la polimerasa para la detección del virus de influenza aviar.

Se desarrolló una prueba transcriptase reversa—reacción en cadena por la polimerasa en tiempo real (de sus siglas en Inglés RRT-PCR) empleando sondas específicas, las cuales al ser hidrolizadas emiten fluorescencia, para la detección del virus de influenza aviar y los subtipos H5 y H7. Se diseñaron iniciadores y sondas específicas para amplificar regiones del gen de la matriz de los virus de influenza aviar, el cual presenta secuencias conservadas entre los diferentes virus de influenza tipo A. Los iniciadores y la sonda para la hemoaglutinina H5 y H7 están dirigidos a las regiones del gen de la hemoaglutinina conservadas entre los virus de influenza aviar de América del Norte. La sensibilidad y especificidad de la prueba RRT-PCR fue comparable a la del aislamiento viral en embriones de pollos en 1550 muestras de hisopos de 109 centros de mercadeo en Nueva York y Nueva Jersey. La prueba de RRT-PCR detectó el virus de influenza en muestras obtenidas en 61 de 65 (93.8%) centros de mercadeo de aves vivas, las cuales sirvieron como fuentes de muestras positivas al aislamiento viral. De los 58 centros de mercadeo positivos a influenza H7 mediante la prueba de inhibición de la hemoaglutinación, 56 (96.5%) fueron positivos al virus de influenza H7 mediante la prueba de RRT-PCR. En este estudio se obtuvieron muy pocas muestras H5 positivas para poder validar esta prueba. Aunque la prueba RRT-PCR fue menos sensible que el aislamiento viral, con base en muestras individuales, se

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demostró que esta prueba para los virus de influenza aviar y para los virus H7 constituyen unas buenas herramientas para la evaluación rápida de lotes en centros de mercadeo de aves vivas.

Key words: avian influenza, live-bird markets, real-time RT-PCR

Abbreviations: AIV = avian influenza virus; BHI = brain heart infusion; CAF = chorioallantoic fluid; CRBC = chicken red blood cells; HI = hemagglutination inhibition; HP = high pathogenicity; LBM = live-bird market; LP = low pathogenicity; NI = neuraminidase-inhibition; PBS = phosphate buffered saline; RRT-PCR = real-time reverse transcriptase-polymerase chain reaction; VI = virus isolation

Avian influenza virus (AIV) has persisted in urban live-bird markets (LBMs) in the northeast, including New York and New Jersey, since the mid-1990s (5,6). Although the H7 AIVs that are currently circulating in the LBMs are characterized as low pathogenicity (LP) based on standard pathotyping tests, there is a risk that these viruses will mutate to high pathogenicity (HP). High-pathogenicity AIVs in poultry have previously occurred in regions where LP viruses had persisted in the poultry population for a period of time, such as in Pennsylvania in 1983, Mexico in 1995, and Italy in 1999 (1,2,3,4). Additionally, the presence of influenza in the LBMs presents a risk of spread to large commercial poultry operations, which was thought to have occurred in 1997–98 and 2001–02 in Pennsylvania (9, data not shown).

Presently, AIV in the LBMs is monitored by routine surveillance that relies on virus isolation (VI) in embryonated chicken eggs for virus detection. Although VI is the gold standard for AIV detection, results are routinely not obtained for 1 to 2 weeks. A more rapid method for the identification of positive markets is necessary for successful eradication and would greatly aid surveillance. We have developed real-time reverse transcriptase-polymerase chain reaction (RRT-PCR) as a rapid method for the detection of type A influenza virus and for further characterization of positive samples with RRT-PCR for the H5 and H7 subtypes.

MATERIALS AND METHODS

LBM sampling. As part of an epidemiologic study, samples were obtained from 109 LBMs in New York and New Jersey. Each sample contained a pool of either cloacal, tracheal, or environmental swabs collected in brain heart infusion (BHI) broth. Tracheal and cloacal swabs were obtained from five birds of each lot (different birds of the same species and from the same source that entered the market at the same time) present at the time the market was sampled, except ducks, from which only cloacal swabs were obtained. Five environmental swabs per sample tube were taken in the following areas in each market: the office, bird area, slaughter area, and red meat area if present.

All samples were tested by both VI and RRT-PCR for the presence of influenza virus. Samples positive for influenza by RRT-PCR were subsequently tested by RRT-PCR for the H7 subtype. H7 negative samples were then tested for H5. The subtypes of all VI positive samples were determined by hemagglutination-inhibition (HI) assay.

Results of the RRT-PCR assay were compiled with the results of VI and subtyping by an independent third party to prevent bias.

Real-time reverse transcriptase/polymerase chain reaction. RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA). The Qiagen one-step RT-PCR kit (Qiagen Inc., Valencia, CA) was used with a 20 μl reaction volume with the following conditions: 10 pmol each primer, 0.3 μM hydrolysis probe, 3.75 mM $MgCl_2$, and 2.5 units RNase inhibitor (Promega, Madison, WI). The primer and probe sequences are in Table 1. All hydrolysis probes were labeled at the 5′ end with 6-carboxyflourescein (FAM) as the reporter dye and at the 3′ end with 6-carboxytetramethylrhodamine (TAMRA) as the quencher dye. The RT step conditions for all primer sets were 30 min at 50°C, 94°C for 15 min. PCR cycling conditions are in Table 2. All temperature transition rates were set to the maximum of 20. Fluorescence data was acquired at the end of each annealing step.

RRT-PCR was performed with the ruggedized advanced pathogen identification device (RAPID) thermocycler (Idaho Technologies, Salt Lake City, UT). Positive and negative results of RRT-PCR reactions were determined by the RAPID auto analysis software and rechecked manually.

Virus isolation and subtyping. Virus isolation was performed in embryonated chicken eggs. Antibiotics and antimycotics were added to each sample in the following final concentrations: 10,000 IU/ml penicillin G, 2000 μg/ml streptomycin, 1000 μg/ml gentamycin, 650 μg/ml kanamycin, and 20 μg/ml amphotericin B. Four 9-to-11-day-old chicken embryos were each inoculated with 300 μl of sample with antimicrobials by the chorioallantoic sac route. The eggs were incubated for 4 days and candled daily for

Table 1. RRT-PCR primer and probe sequences.

Specificity	Primer/probe name	Sequence
Influenza A	M+25	5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′
	M–124	5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′
	M+64	5′- FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3′
Avian H5	H5+1456	5′-ACG TAT GAC TAT CCA CAA TAC TCA G-3′
	H5–1685	5′-AGA CCA GCT ACC ATG ATT GC-3′
	H5+1637	5′-FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA-3′
Avian H7	H7+1244	5′-ATT GGA CAC GAG ACG CAA TG-3′
	H7–1342	5′-TTC TGA GTC CGC AAG ATC TAT TG-3′
	H7+1281	5′-FAM-TAA TGC TGA GCT GTT GGT GGC A-TAMRA-3′

viability. Embryos dying within 24 hr of inoculation were discarded as nonspecific. Chorioallantoic fluid (CAF) was tested for hemagglutination of 0.5% chicken red blood cells (CRBCs) in phosphate buffered saline (PBS). Samples negative for hemagglutination were passaged a second time.

Hemagglutinin- and neuraminidase-inhibition tests with subtype-specific antisera were used to subtype all hemagglutination-positive samples. For the hemagglutination-inhibition test (HI), CAF was standardized to four HA units and HA mixed with an equal volume of influenza subtype reference serum at a titer between 1:32 and 1:64. Reference serum and CAF were incubated for 30 min at room temperature, and 0.5% CRBCs were added and mixed. The assay was evaluated for HI after incubation at room temperature for 30 min. The microneuraminidase-inhibition test (NI) was performed as previously described (8).

RESULTS

A total of 1550 samples from 109 LBMs were tested for AIV by both RRT-PCR and VI. Two-hundred two samples (13%) were positive and 1183 (76.3%) samples were negative by both VI and RRT-PCR for the presence of AIV. There were 165 (10.6%) samples that were positive for the presence of AIV by only one method; 64 samples were only positive by VI and 101 samples were positive by only RRT-PCR.

Sixty-one markets (55.9%) were positive for the presence of AIV by both VI and PCR. AIV was not detected in 37 (33.9%) of the markets, and AIV was detected by only one of the methods in 11 (10%) of the markets. RRT-PCR detected AIV in 61 of the 65 (93.8%) markets that were positive by VI.

Of the 202 samples that were positive for AIV, 194 were determined to be the H7 subtype and 1 was determined to be the H5 subtype by both HI and RRT-PCR. Three samples were determined to be H7 by HI only, and one sample was determined to be H7 by RRT-PCR only.

The H7 subtype was detected in 56 (51.3% of all tested markets or 91.8% of the positive markets) markets by both HI and RRT-PCR. Overall RRT-PCR detected the H7 subtype in 56 of the 58 markets (96.5%) that were positive for H7 by HI. The H5 subtype was detected in one (0.9%) market and was determined to be H5 by both HI and RRT-PCR.

DISCUSSION

RRT-PCR was chosen as an alternative method of AIV detection because it offers advantages over both VI and standard RT-PCR. The primary advantage of RRT-PCR over VI for detection of AIV, particularly for the application of screening flocks and LBMs, is speed. The results of AIV detection and subtyping may be obtained within 1 day as compared to weeks with VI. Another benefit of RRT-PCR is that there is reduced handling of potentially infectious material; the virus is inactivated during RNA extraction. The advantages of RRT-PCR over standard RT-PCR include speed and the reduced chance of cross-contamination among samples because no postamplification sample handling is necessary. Additionally, the labeled probe used to detect the PCR product with real-time PCR methods is target specific, providing an additional level of confirmation that the PCR product is the expected target, as compared to standard RT-PCR.

Table 2. RRT-PCR protocols.

Target	Protocol
Influenza MA gene	45 cycles of 0 sec, 94°C; 20 sec, 60°C
H5	40 cycles of 0 sec, 94°C; 20 sec, 57°C; 5 sec, 72°C
H7	40 cycles of 0 sec, 94°C; 20 sec, 58°C

Although RRT-PCR is a fast, sensitive, and specific method when compared to VI, it must be taken into account that the methods measure different things. Virus isolation detects viable virus, whereas RRT-PCR detects intact viral RNA. Therefore, RRT-PCR may detect defective particles and virus that has been inactivated during handling. Conversely, sequence variation, RNA degradation, and the presence of PCR inhibiting substances in the sample, could prevent detection by RRT-PCR but possibly not by VI. This may help to explain the 10.6% of the samples that were positive for the presence of AIV by only one assay. A final consideration regarding the differences in detection between VI and RRT-PCR may be technical and related to the efficiency of RNA extraction, RNA degradation, the efficiency of the reverse transcription step, and the sample volume used (VI uses an equivalent of nine times more of the original sample that each RRT-PCR reaction).

The presence of AIV in 55.9% of the markets tested, of which 91.8% were positive for H7, is further evidence for the persistence of AIV in the LBM system. At present it is not clear how the virus continues to circulate in the LBMs, although molecular epidemiological studies have shown that it is likely that at least the H7 subtype viruses may have originated from a single introduction of the virus (7). The presence of hemagglutinin subtypes H5 and H7, which are associated with high-pathogenicity influenza in poultry, support the need for eradication of the virus from the markets and continued surveillance in case of reintroduction of the virus in the markets.

This study evaluated RRT-PCR as a rapid screening method for the detection of AIV in LBMs or flocks by comparison to VI. RRT-PCR performed well for the detection of AIV positive LBMs when compared with VI, although there was some difference in detection between the two assays with individual samples. Furthermore RRT-PCR and HI yielded similar results for detection of the H7 hemagglutinin subtype by both individual sample and by market. Finally, the incidence of H5 was too low to conclusively evaluate this assay versus VI.

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Erratum

In the article, ''Development of Real-Time RT-PCR for the Detection of Avian Influenza Virus'' by E. Spackman, D. A. Senne, L. L. Bulaga, T. J. Myers, M. L. Perdue, L. P. Garber, K. Lohman, L. T. Baum, and D. L. Suarez, which appeared in Avian Diseases 47 (Suppl.): 1079–1082, there was an error. The last sentence of the first paragraph in the Results section should state, ''There were 165 (10.6%) samples that were positive for the presence of AIV by only one method: 101 were only positive by VI and 64 were positive by only RT-PCR.''